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RESISTANCE OF A TUNICATE TO FOULING

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Although sessile organisms of several phyla are believed to have defenses against epizooic recruitment (fouling), the ecological consequences of epizooic recruitment have received relatively little attention. In simple ascidians, such as *Ascidia nigra,* (Savigny) extensive fouling could have several detrimental effects: first, obstruction of the siphon apertures could prevent feeding and respiration; secondly, over-topping could reduce feeding efficiency by reducing water movement past the ascidian's siphons; thirdly, epibionts could compete with the ascidian for food; fourth, a heavy load of fouling organisms could also increase sedimentation, which could be detrimental; and finally, increased drag due to epibionts could make an ascidian more likely to be dislodged by water movements. However, fouling may have a beneficial effect by providing camouflage.

A. nigra is a common member of fouling communities in the West Indies and Bermuda. This ascidian is free of epibionts, although sedentary organisms settle and grow on the tests of many other ascidians (Goodbody, 1962; Stoecker, unpublished observations). Although they may survive from 18 to 22 months (Millar, 1971), mature *A. nigra* never are overgrown by other epibenthic species. Goodbody and Gibson (1974) noted that, in Jamaica, adult populations of this species were relatively free of competitors and predators. Goodbody (1962) attributed the lack of epizooic recruitment on *A. nigra* to the sloughing off of the test surface.

The ability of ascidians to concentrate vanadium from seawater is well known but poorly understood (Goodbody, 1974; Carlson, 1975; Carlisle, 1968; Danskin, 1978; Webb, 1939, 1956). Vanadocytes are abundant in the blood of *A. nigra.* Partially-complexed vanadium is localized within vacuoles of vanadocytes (Kustin, Levine, McLeod, and Curby, 1976). These vacuoles, called vanadophores, also contain sulfuric acid (Webb, 1939). Vanadocytes are known to migrate through blood vessels into the test. Many ascidians, including *A. nigra,* have sulfuric acid-filled capsules called bladder cells in their tests. The formation of bladder cells is believed to be associated with the degeneration of vanadocytes (Goodbody, 1974).

Vanadium, except in trace amounts, is a metabolic poison (National Research Council, 1974) and is expected to be toxic to a wide variety of organisms. Similarly, strongly acidic solutions are destructive to most tissues. The present study investigates the role of these two inorganic toxins in the prevention of recruitment of *Ecteinascidia turbinata,* an ascidian, and *Pennaria tiarella,* a hydroid, onto the test of *A. nigra.* Both *E. turbinata* and *P. tiarella* produce larvae throughout the summer and are found in the same habitats as *A. nigra* in Bermuda.

Although the roles of organic allelochemicals are known in some organisms

(Whittaker and Feeny, 1971), inorganic toxins have received little attention. Allelochemicals in marine organisms may be effective against parasites and epibionts (Siebruth, 1968; Siebruth and Conover, 1965; Fenical, 1975), competitors (Jackson, 1977; Jackson and Buss, 1975), and predators (Bakus, 1970; Bakus and Green, 1974).

Many functions for vanadium and vanadocytes in ascidians have been proposed; these include oxygen transport (Carlson, 1975), tunicin synthesis (Endean, 1961), phagocytosis of foreign bodies (Brown and Davies, 1971; Wardrop, 1970), cellular immunity (Anderson, 1971) and production of sulfuric acid for an unknown purpose (Swinehart, Briggs, Halko, and Schroeder, 1974). However, there is no general agreement on the functions of vanadium or vanadocytes in ascidians (Webb, 1956; Carlisle, 1968; Carlson, 1975). The present paper proposes that vanadocytes, at least in some species, play a role in chemical defense against fouling organisms.

Materials and Methods

Collection and maintenance of specimens

During the summer of 1977, *A. nigra* individuals between 4 and 12 cm long were collected from bridge pilings, docks, and coral rubble in the vicinity of the Bermuda Biological Station at St. George's, and near the Bermuda Government Aquarium at Flatts, Bermuda. Specimens were maintained in running seawater tanks at the Bermuda Biological Station at ambient temperature and salinity.

P. tiarella stalks and *E. turbinata* colonies were collected in the same habitats and locations as *A. nigra*. Mature *E. turbinata* colonies release larvae when the colonies are transferred to fresh bowls of seawater. *E. turbinata* larvae were collected and used in experiments within five hours of their release.

P. tiarella stalks were collected in the afternoon. For spawning, three female stalks and one male stalk were placed in 200 ml of filtered (0.45 μm Millipore filter) seawater in a Cambridge bowl. The bowls were placed in the dark at 6 PM. Four hours later, the released eggs were transferred to fresh bowls of filtered seawater. In the morning, the unfertilized eggs were removed from the culture bowls. *P. tiarella* planulae were used in experiments on the second day after spawning.

Chemical analyses and pH determinations

Tests of *A. nigra* individuals were removed and rinsed in seawater. The part of the test where the organism had been attached contains large blood vessels and was discarded. The black surface deposit on the test was collected by brushing the external surface in distilled water, washed, and concentrated by centrifugation (Sorval RC-5 centrifuge, GSA head, 10,000 rpm, 15 min). The surface deposit, test, and soft bodies (animals with the tests removed) were weighed and then dried at 60° C. Dried samples were weighed, digested in hot $HNO_3 : H_2SO_4 : : 2 : 1$ in a reflux system, and analyzed for vanadium with an atomic absorption spectrometer using the method of standard additions (Kustin *et al.*, 1976).

The pH of the tunic and body surfaces was determined with a miniature pH probe (Microelectrodes Inc., Londonderry, N.H.).

Morphological analyses

Thin cross-sectional slices of tunic (less than 1 mm) were taken with razor blades, fixed in three percent glutaraldehyde in seawater, and embedded in araldite 506 epoxy resin. Photomicrographs were taken of unstained slices in the embediment to show the distribution of pigment cells. Fresh slices were also stained with neutral red in seawater and examined microscopically to study the distribution of bladder cells in the test.

Larval settlement experiments

Larval settlement experiments were run in Cambridge bowls containing 200 ml of filtered seawater. Twenty-five to thirty-five larvae of *P. tiarella* or *E. turbinata* were added to each replicate bowl. Larval responses to experimental and control substrates were assessed by counting larvae on each substrate. Larvae were examined under low magnification (30X) using a compound scope and classified as settled, active, inactive (no response to prodding or to the switching on and off of the sub-stage illuminator) or dead (evidence of decay). Stolon length of *E. turbinata* was measured with an ocular micrometer.

In the first set of experiments, the tunic of *A. nigra* was tested for anti-fouling properties. Discs 2 cm in diameter were cut from *A. nigra*'s test; control discs were cut from control agar (3% in seawater) darkened with lampblack to control for color. (Lampblack in agar has no effect on *P. tiarella* or *E. turbinata* larvae). Test discs were alternated with control discs (three each) embedded in a control agar substrate at the bottom of replicate Cambridge bowls. Larval response was scored after 19 to 22 hr.

In the second set of experiments, an aqueous extract of *A. nigra*'s test was assayed for anti-fouling properties. The extract was prepared by mixing pieces of fresh test with an equal weight of distilled water in a Waring blender. The resulting mixture was brought to a boil and then filtered through a Whatman #1 filter. The vanadium content of the extract was 23 ppm. The extract had a pH of 2.0. An aliquot of the extract was neutralized with 1 N NaOH.

Cambridge bowls were prepared with one half of the bottom covered by control agar and the other half covered by extract agar. Agars made with the unneutralized extract would not set unless the agar was first dissolved in distilled water (approximately 10 ml per gram of agar) before the extract was added. Larvae were added to the bowls and after three hours they were scored and counted.

In the third set of experiments, the effects of vanadium alone on *P. tiarella* and *E. turbinata* were tested. Vanadium was added as vanadyl sulfate to agar. Sulfuric acid was added to the control agar to obtain a sulfate ion concentration equivalent to that found in the experimental agar with the highest vanadium concentration. Vanadium and control agars were neutralized with 1 N NaOH. Replicate bowls were prepared for each concentration of vanadium tested. Larvae were scored after 16 hr exposure.

TABLE I

Distribution of vanadium in Ascidia nigra.

Sample	N	ppm Vanadium ± SD	
		Wet weight basis	Dry weight basis
Without tunic	8	144.81 ± 72.69	—
Tunic	6	6.00 ± 4.49	95.78 ± 69.91
Surface deposit	4	253.32*	1021.92 ± 101.21

* Calculated from ppm V dry weight basis.

RESULTS

Chemical analyses and pH determinations

In *A. nigra,* vanadium is not evenly distributed between the test and the soft body parts (test removed) (Table I). The soft body contains about 145 ppm vanadium (wet weight basis), whereas the test contains only 6 ppm (wet weight basis). Ciereszko, Ciereszko, Harris, and Lane (1963), observed a similar distribution of vanadium in *A. nigra.* However, the black surface deposit brushed from the test's surface has a very high vanadium content of approximately 253 ppm (wet weight basis). Vanadium is concentrated in the black surface layer relative to the test as a whole.

The external surface of the test releases a strong acid when it is even slightly bruised (Table II). This acidity is believed to be due to the release of free sulfuric acid from capsules in the test (Webb, 1939; Andrew, 1961; Goodbody, 1974).

Morphological analyses

Black pigment is concentrated near the surface of *A. nigra*'s test, which is several millimeters thick. The deeper layers of the test are semi-transparent. In sections of the test, black cells are abundant, particularly near the outer surface (Fig. 1). These black test cells are probably the same as the blue cells described by George (1930) and the pigment granules described by Hecht (1918) and Goodbody (1962) in *A. nigra*'s test. Test cells have been described in tests of other ascidians which concentrate vanadium or iron (Stievenart, 1970; Smith, 1970). The test cells in *A. nigra* are believed to be derived from vanadocytes, which migrate from blood vessels into the test matrix, where the vanadocytes turn blue-black and begin to degenerate (Hecht, 1918; Berrill, 1950; Goodbody, 1962;

TABLE II

Acidity of Ascidia nigra.

Sample	N	pH ± SD
Without tunic	6	7.48 ± 0.13
Tunic	6	1.96 ± 0.62

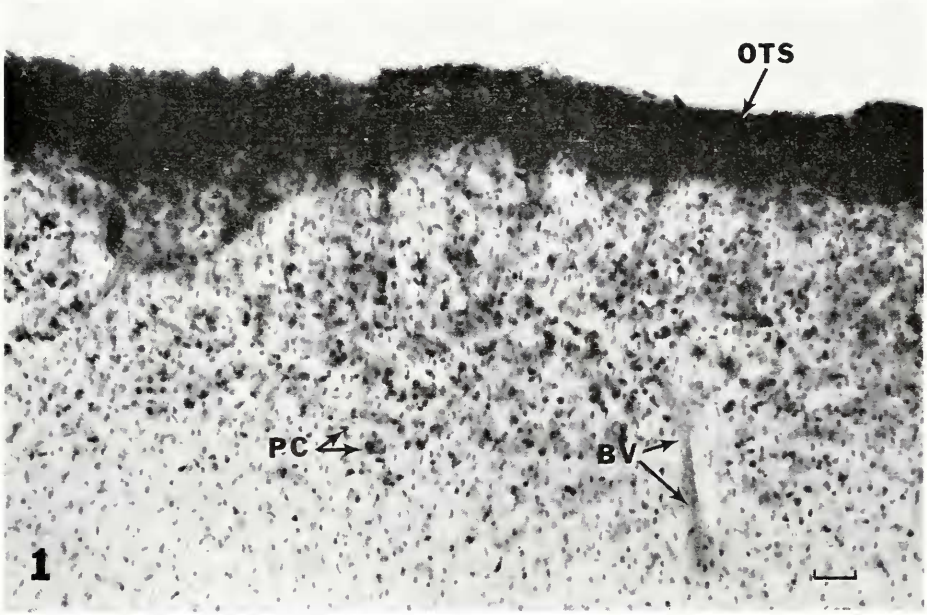

FIGURE 1. Cross-section of *Ascidia nigra* test showing concentration of pigment (black test cells) near outer surface of test. Magnification bar = 100 μ. Abbreviations are: OTS, outer test surface; PC, pigment; and BV, blood vessel.

Smith, 1970). The nuclei of test cells, even those found near the test surface, stain red with Feulgen's stain, indicating that these cells were alive prior to fixation (unpublished observations, D. Stoecker). It is reasonable to propose that the test cells, which are derived from vanadocytes, form the black surface layer described by Goodbody (1974). The derivation of the test cells from vanadocytes would explain the high concentration of vanadium found in the black surface deposit on the test.

Bladder cells, because of their acid contents, stain cherry red with neutral red

TABLE III

Effect of tunic surface on Pennaria *(19–22 hours).*

Treatment	Number of larvae		
	Active	Inactive	Dead
External surface of tunic	1	0	71
Internal surface of tunic	5	34	22
Control	2	21	0

$N = 156$
$G = 124.65$
$P < 0.005$

TABLE IV

Effect of tunic extract on Pennaria *(3 hrs).*

Agar surface	Number of larvae			
	Active	Inactive	Dead	
Extract (pH 2)*	0	11	14	$N = 40$
Control (pH 6)	13	2	0	$G = 41.762$
				$P < 0.005$
Extract (pH 7)*,**	9	3	0	$N = 25$
Control (pH 6)	13	0	0	$G = 1.826$
				n.s.

* 22.8 ppm vanadium.
** Neutralized with 1 N NaOH.

in seawater. Bladder cells were abundant in the test but were concentrated just beneath the outer border of the test.

Larval settlement experiments

Table III summarizes the results of several settling experiments designed to assay the response of *P. tiarella* to pieces of *A. nigra*'s test. *P. tiarella* larvae accumulated on test discs relative to control discs. A greater proportion of the *P. tiarella* on test discs than on control discs were inactive or dead. *P. tiarella* larvae probably accumulated on test discs as they were inactivated by the toxic effects of contact with the test.

Contact with the extract agar (pH 2, 23 ppm vanadium) inactivated *P. tiarella* larvae within 3 hr (Table IV). Neutralized extract agar had no significant effect on larvae within the three-hour exposure period (Table IV).

Vanadium had a toxic effect on *P. tiarella* larvae (Table V). Concentrations of 250 ppm (wet weight basis), or more, of vanadium in the substrate significantly

TABLE V

Effect of vanadium on Pennaria *(16 hrs).*

ppm Vanadium	Number of larvae				
	Settled	Active	Inactive	Dead	
0	1	9	13	0	
25	4	7	12	1	
50	2	9	9	1	$N = 153$
100	3	7	18	2	$G = 119.484$
250	0	1	30	1	$P < 0.005$
500	0	0	12	11	

A posteriori tests:

ppm Vanadium	0	25	50	100	250	500

TABLE VI

Effect of tunic surface on Ecteinascidia *(19–22 hours).*

Treatment	Number of larvae			
	Settled	Active	Inactive	Dead
External surface of tunic	4	11	8	6
Control	27	15	11	3

$N = 85$
$G = 12.52$
$P < 0.01$

increased the proportion of *P. tiarella* larvae which were inactive or dead relative to control agar substrates.

E. turbinata larvae, which swim rapidly but erratically until metamorphosis, were not found in greater numbers on the test discs than on the control discs (Table VI). However, a greater proportion of the larvae on test discs were inactive or dead than on control discs.

The extract agar substrate (pH 2, 23 ppm vanadium) rapidly killed *E. turbinata* larvae (Table VII). Neutralized extract agar had no effect on *E. turbinata* within a three-hour exposure period, but within 16 hr a deterimental effect was evident (Table VII).

Concentrations of 250 ppm, or more, of vanadium significantly decreased stolon length in *E. turbinata* (Table VIII). Stolon length should be a good indicator of settlement success.

TABLE VII

Effect of tunic extract on Ecteinascidia.

Agar surface	Number of larvae		
	Active or Settled	Inactive or Dead	
	(3 hours)		
Extract (pH 2)*	6	27	$n = 64$
Control (pH 6)	25	6	$G = 26.906$
			$P < 0.005$
	(3 hours)		
Extract (pH 7)*,**	20	4	$n = 57$
Control (pH 6)	28	5	$G = 0.024$
			n.s.
	(16 hours)		
Extract (pH 7)*,**	15	16	$n = 48$
Control (pH 6)	14	3	$G = 6.768$
			$P < 0.01$

* 22.8 ppm vanadium.
** Neutralized with 1 N NaOH.

TABLE VIII

Effect of vanadium on Ecteinascidia *metamorphosis (16 hrs).*

ppm Vanadium	N	Mean stolon length (mm)	
0	10	0.53	
50	2	0.44	
100	5	0.52	$F_s = 11.958$
250	5	0.24	$P < 0.001$
500	9	0.20	
1000	2	0.10	

A posteriori tests:

Source of variation	SS	
0 vs 50 vs 100	0.01415	n.s.
250 vs 500 vs 1000	0.02800	n.s.
(0 + 50 + 100) vs (250 + 500 + 1000)	9.63166	$P < 0.001$

DISCUSSION

The results presented here demonstrate that the test of *A. nigra* is toxic to *P. tiarella* and *E. turbinata* larvae. There was no evidence that these larvae avoided the test surface. Similar larval response to a toxic substrate was obtained by Wisely (1962), who found that ectoproct larvae accumulated on surfaces covered with anti-fouling paint even though larvae were killed by contact with the paint. Instances of larval selectivity for settling substrates based on physical factors such as light, temperature, water currents, and contour, texture and angle of the surface are well documented (Osman, 1977). Biological factors, such as presence of a microbial film, type of algae, and presence of the same species, also affect larval settlement (Osman, 1977). However, few data are available on the response of settling larvae to toxic substrates (Wisely, 1962, 1963a, b, 1964).

To understand the toxicity of *A. nigra's* surface, both chemical and morphological characteristics of *A. nigra's* test must be considered. The explanation proposed here is that the vanadium-rich deposit on *A. nigra's* surface is formed by degenerating vanadocytes and prevents epizooic recruitment through its toxicity and by its tendency to slough. The vanadium-agar substrate experiments demonstrated that vanadium alone can inactivate or kill settling larvae. The black surface deposit may partially explain why *A. nigra* is free of epibionts while many other solitary ascidians are fouled.

The external surface of *A. nigra's* test releases a strong acid when it is even slightly injured or bruised. The capsules in the test, which are believed to be the source of the free acid, are concentrated near the test surface. This location would make the capsules effective against organisms which may try to establish a holdfast on the test by penetrating the surface with stolons or other anchoring devices. It is possible that the capsules discharge acid to the test's surface even in the absence of injury (Goodbody, 1974).

Vanadocytes and many of the black test cells are probably alive and may move to sites of injury in the test. The disturbance of the test surface by holdfasts of

settling larvae may cause injury. Thus, vanadium and acid may be preferentially released at sites where these toxins are most needed to defend *A. nigra* against fouling and overgrowth.

Not all ascidians which concentrate vanadium or which produce free sulfuric acid are resistant to fouling. For example, *Ascidiella aspersa* concentrates vanadium and has acid-filled test capsules (Webb, 1939; Stievenart, 1970) but lacks typical vanadocytes (Webb, 1939; Berrill, 1950). This species is often covered by epibionts (Berrill, 1950; Stievenart, 1971). Although vanadium is present in this species, there is no evidence that it is concentrated at the test surface. The acid capsules in *A. aspersa* are concentrated in the lower layers of the test (Stievenart, 1970), which may explain why epibionts can attach to the test.

Rhopalaea birkelandi and *Halocynthia aurantium,* like *A. nigra,* are simple ascidians which are resistant to epizooic recruitment (Tokioka, 1971; Smith, 1970). The resistance of these species to fouling has not been investigated. *R. birkelandi* is very acidic (Birkeland, personal communication) and also has a high vanadium content (D. Stoecker, unpublished data). Geranyl hydroquinone has been isolated from *Aplidium* sp. by Fenical (1976), and it is possible that this or other organic toxins, in addition to inorganic toxins, may be involved in chemical defense in some ascidians.

If fouling is often detrimental to epibenthic organisms, one may ask how species which are susceptible to epizooic recruitment avoid the difficulties imposed by fouling. Some ascidians are sexually mature at three weeks, whereas other species may grow for over a year before they are sexually mature (Jackson, 1977). The fast-maturing species may become senescent before fouling organisms interfere with their growth. Therefore, fouling may be inconsequential for these species. Some colonial species, for example *E. turbinata,* periodically die back to stolons and then produce a new generation of zooids from the stolons (Morgan, 1977). *E. turbinata,* although it concentrates vanadium (Ciereszko *et al.,* 1963), is susceptible to fouling (D. Stoecker, unpublished observations). Die-backs may periodically rid colonies of their epibionts.

Vanadium and free acid may be involved in allelochemical interactions between ascidians and their parasites and predators as well as their epibionts and competitors. Extracts of *A. nigra* inhibit the growth of bacteria; Burkholder (1973) believes this effect is due to acidity. Vanadocytes of *Molgula manhattensis* encapsulate large foreign bodies and are involved in graft rejection (Anderson 1971). Anderson has suggested that vanadocytes which invade grafts may rupture and release their vacuolar contents, thus killing the graft. Vanadocytes accumulate at sites of injury (Anderson, 1971; Wardrop, 1970) and may possibly encapsulate or destroy invading organisms.

Newly metamorphosed specimens of *A. nigra,* once they develop the black surface deposit, have low mortality until senescence. Goodbody and Gibson (1974) partially attribute this population stability to lack of predation. The test substance, but not the soft body of *A. nigra,* is distasteful to fish (Goodbody, 1962). Haynes, Sangster, Steven and Thomas (1967) observed that extracts of *A. nigra's* test evoked an avoidance response from fish. Acidity may alone or partially explain the distastefulness of *A. nigra.* Acidic tissues are repellant to most fish (Thompson, 1960). Webb (1939) suggested that the acid-filled capsules in the tests of

some ascidians may discourage predator attack. However, some types of ascidians which concentrate vanadium but which are not highly acidic, such as *E. turbinata* and *Clavelina picta,* also seem to be resistant to most general predators (D. Stoecker, unpublished data). Perhaps vanadium or other inorganic or organic chemicals in these ascidians can protect them from predation.

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Summary

Ascidia nigra is free of epibionts, although many other ascidians are susceptible to epizooic recruitment. Larvae of *Pennaria tiarella* and *Ecteinascidia turbinata,* two epibenthic species found in the same habitats in Bermuda as *A. nigra,* were used in laboratory experiments which demonstrated that the surface of *A. nigra's* test is toxic to settling larvae. There was no evidence that *P. tiarella* or *E. turbinata* is repelled by the toxic surface. The toxicity of *A. nigra* is probably due to the high vanadium content of the surface deposit and to the release of free sulfuric acid from capsules in the test. Both the vanadium-rich surface deposit and the acid-filled capsules are believed to be formed by degenerating vanadocytes in the test. Vanadocytes may also be involved in defense against competitors, parasites, and predators of ascidians.

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